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14. ABSTRACT

The purpose of this study was to investigate the hypothesis that PTEN null prostate cancer cells and tumors will exhibit increased radiosensitivity to inhibition of the DNA repair enzyme, PARP, compared to wild-type PTEN-expressing cells and tumors, resulting in increased efficacy of radiotherapy and chemotherapy. The specific objectives of this study were to (1) determine the effect of PARP inhibition on the cellular response to ionizing radiation or docetaxel in DU145 PTEN wild-type vs. PC-3 PTEN-null cells. and (2): determine the efficacy of PARP inhibition in combination with radiotherapy or docetaxel in DU145 and PC-3 prostate cancer xenograft models. Radiosensitization was measured in DU145 cells and PC-3 cells by γ H2AX foci formation and disappearance, quantitation of olive tail moment in comet assay, clonogenic cell survival and apoptosis assay. γ H2AX foci assays revealed that ABT888 in combination with radiation therapy (RT) increased the level of DNA damage compared to drug alone and RT alone in both cell lines, and that the combination inhibited DNA repair in PC-3 cells but not in DU145 cells. Apoptosis assays indicated that DU145 cells were more susceptible than PC-3 cells to apoptosis induction by monotherapy with ABT888, docetaxel or RT. However, triple modality treatment with ABT888, docetaxel and RT increased apoptosis similarly in both cell lines. Clonogeic assays revealed that although DU145 was more radioresistant than PC-3 cells, ABT888 similarly radiosensitized both cell lines. It is tentatively concluded that radiosensitization by ABT888 is independent of PTEN status in these cells.

15. SUBJECT TERMS PC-3, DU145, PTEN, radiosensitization, ABT888, Docetaxel

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Introduction

This proposal specifically focuses on the PTEN tumor suppressor and its interaction with the poly (adenosine diphosphate [ADP]–ribose) polymerase (PARP) DNA repair pathway in advanced prostate cancer (APC). It examines the hypothesis that PTEN deficiency induces chemo/radioresistance in APC that can be specifically overcome through PARP inhibition. This hypothesis was based on the observation that PTEN affects double-strand breaks through regulation of Rad51, a key player in homologous recombination repair (HRR) of DNA double strand breaks (DSBs).[1] It was speculated that PTEN null cells have DNA repair deficiencies in the HRR pathway that will compromise their ability to repair DNA DSBs and will therefore be highly sensitive to PARP inhibition of DNA repair. Several measures of DNA damage and repair were proposed to assess radiosensitization in vitro in PTEN wild-type (DU145) vs. PTEN-null (PC-3) cells. In vivo studies were proposed to determine the efficacy of PARP inhibition in combination with radiotherapy or docetaxel in PTEN wild-type and PTEN-null human prostate cancer xenograft models implanted in nude mice.

Body

Task 1: Determine how PTEN status impacts the response to DNA damage following chemo/radiation in the absence or presence of PARP inhibition

1a. γH2AX assay. The number of DNA double strand breaks (DSBs) was assessed by quantification of γH2AX foci based on a previously published protocol[2]. Briefly, fixed cells were analyzed for γH2AX 30 min and 24 hrs following treatments. Primary anti-phospho-γH2AX mouse monoclonal antibody was added at a dilution of 1:300 in 5% bovine serum albumin (BSA). Secondary Alexa Fluor 594 donkey anti-mouse antibody (Invitrogen Molecular Probes, Eugene OR) was added at a dilution of 1:500 in 5% BSA. Cells were counterstained with DAPI incorporated into ProLong Gold mounting medium (Invitrogen, Molecular Probes, Eugene OR) for nuclei visualization. γH2AX foci visualization was performed on a Zeiss LSM 510 Meta Confocal Microscope (Carl Zeiss Microscope Inc., Thornwood, NY) using a 40X oil immersion lens and analyzed by Image J software provided by NIH.
Assays were performed 30 min and 24 hrs following radiation in order to measure maximum and residual damage (i.e., following DNA repair)

Results:

Figure 1 indicates that ABT888 alone induces more immediate DNA damage in PC-3 vs. DU145 cells, and inhibits DNA repair in both cell lines. Radiation (RT) alone induced less initial DNA damage in PC-3 cells vs. DU145 cells; however, repair was seen in DU145 but not in PC-3. The addition of ABT888 to RT increased the level of DNA damage compared to drug alone and RT alone in both cell lines. The combination inhibited DNA repair in PC-3 cells but not in DU145 cells.

Effect of Docetaxel on gamma H2AX assay: Docetaxel at a concentration of 5 nM induced apoptosis in both PC-3 and DU145 cells which made it difficult to assess for γH2AX foci induction in the largely apoptotic nuclei. Therefore experiments with docetaxel were not analyzed for γH2AX foci .

1b. Comet assay. The kinetics of DNA DSBs and repair was assessed by measuring olive tail moment using Trevigen's CometAssay kit under alkaline conditions (Gaithersburg, MD). Comet Score image analysis software was used to calculate olive tail moment which is a relative measure of DNA damage.

Results:

Figure 2 - 4 show the response of DU145 and PC-3 cells to radiation doses (0 – 30 Gy). A dose dependent response is evident. Based on these observations, a dose of 15 Gy was selected to test the effect of ABT888 on the kinetics of DNA repair following radiation.

Figures 5-10 indicate that ABT888 + RT enhances initial DNA damage and slows down DNA repair following 15 Gy RT in both cell lines. The kinetics of repair up to 30 min is similar in both cell lines.

This data, is confirmatory with the γ H2AX assay data, since both assays indicate that DNA repair is compromised following ABT888 and radiation. However, the γH2AX foci assay, which was assessed 24 hr following treatment, distinguishes between PC-3 and DU145 cells in that it demonstrated prolonged DNA repair inhibition in PC-3 cells compared to DU145 cells.

Effect of docetaxel: no difference was seen in repair between docetaxel + RT vs radiation alone in both cell lines. The combination of docetaxel plus ABT888 and RT will be tested within the 6 month no-cost extension period.

1c. Cell Survival Assay. Clonogenic cell survival assay was performed as previously described[3], with exponentially growing cells with and without ABT888 treatment for 24 hr. Data was fit to a linear quadratic model for cell survival. The mean +/- SEM from at least three independent experiments were obtained.

Results:

Figure 11 shows ABT888 at 100μM reduces clonogenicity in both cell lines to approximately 60%. Therefore ABT888 at 100μM was chosen to study its radiosensitizing potential.

Figure 12 indicates that DU145 cells are more radioresistant than PC-3 cells, as determined by a smaller alpha (α) value (0.028) (initial slope of curve) than seen in PC-3 cells (α = 0.09). ABT888 acts as a radiosensitizer in both DU145 and PC-3 cells as determined by an increase in α of the survival curves from 0.028 to 0.14 and from 0.09 to 0.36 in DU145 and PC-3 respectively. The sensitizer enhancement ratios calculated as the ratio of the initial slopes (α) of survival curves were not significantly different (5 ± 2 and 4 ± 3.0 for DU145 and PC-3

respectively, p= 0.9) from each other.. Therefore, the relative degree of radiosensitization was similar in both cell lines after ABT888 treatment.

1d and 1e. qPCR and immunoblots. These tasks will be completed within the 6 month no-cost extension period.

1f. Apoptosis Assay. Cells were treated with ABT888 (50 uM) and Docetaxel (5 nM) for 24 hr. Radiation was administered at 6 Gy following drug treatments. Cells were collected 24 hr post radiation for apoptosis evaluation using an FITC Annexin V and propidium iodide kit (BD Pharmangin) and analyzed by flow cytometry (Coulter (XL-MCL). Tabulation of percentage of cells undergoing early and late apoptosis (Figure 12) is based on graph shown in Figure 11.
Results:
Figures 13 and 14 indicate DU145 cells are more susceptible than PC-3 cells to apoptosis after treatment with RT alone, docetaxel alone and ABT888 alone. DU145 cells are also more susceptible than PC-3 cells to apoptosis after treatment with ABT888 + docetaxel and docetaxel + RT. Since PTEN suppresses signaling in the anti-apoptotic PI3K/Akt pathway, the presence of PTEN wildtype in this cell line may explain the tendency to undergo apoptosis more readily than the PTEN null PC-3 cell line. However, both cell lines are equally susceptible to apoptosis following treatment with ABT888 + RT. Furthermore, triple modality treatment with ABT888, docetaxel and RT increases apoptosis in both cell lines.

Task 2. Determine the efficacy of PARP inhibition in combination with radiotherapy or docetaxel in vivo in two human prostate cancer xenograft models

2a. Establish prostate tumor xenografts in nude mice.
PC-3 and DU145 tumor cell suspensions ($1x10^7$ cells in 100 µl phosphate buffered saline) from each tumor cell line are being implanted subcutaneously into the right hind limbs of athymic NCR NUM mice (Taconic Farms, Hudson, NY). Mice are not pretreated before tumor implantation. Tumors are allowed to grow for approximately 3-4 weeks until reaching an approximate volume of 100-150 mm^3 before start of treatment. Tumors are randomized into treatment groups when they reach appropriate size.
Results;
Figure 15 shows tolerability data for the drugs, ABT888 and Docetaxel. The results indicate that at the doses given, there is no toxicity with regard to body weight as an endpoint. Therefore these doses were used in tumored animal studies.
The tumored animal studies are ongoing. Animal studies have been delayed because of slow growth of tumors. They are expected to be completed within the 6 month no-cost extension period.

Key Research Accomplishment

- **ABT888 has been shown to be an effective radiosensitizer in prostate cancer cell lines, DU145 and PC-3 through a number of DNA damage assays, which were all confirmatory.**

Reportable Outcomes
None to date

Conclusion
The PARP inhibitor, ABT888, potentiates radiation-induced damage in DU145 (PTEN wildtype (wt)) and PC-3 cells (PTEN null), It is not clear, however, from the present in vitro data whether PTEN status is important for ABT888-induced radiosensitization. While DNA repair was more compromised in PTEN null cells than in DU145 cells, based on γH2AX assays, the apoptosis and clonogenic survival assays seem to indicate radiosensitization is independent of PTEN status in these cells. Data from completed in vivo animal studies may shed further light on this issue. Further work on isogenic models of PTEN wt and PTEN prostate cancer models may also help clarify this issue.

References

1. Shen WH, Balajee AS, Wang J, Wu H, Eng C, Pandolfi PP, Yin Y. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell, 2007 January 12;128(1):157-70.

2. Munshi A, Tanaka T, Hobbs ML, Tucker SL, Richon VM, Meyn RE. Vorinostat, a histone deacetylase inhibitor, enhances the response of human tumor cells to ionizing radiation through prolongation of gamma-H2AX foci. *Mol.Cancer Ther.* 2006;**5**:1967-74.

3.Wachsberger PR, Lawrence RY, Liu Y, Xia X, Andersen B, Dicker AP. Cediranib enhances control of wild type EGFR and EGFRvIII-expressing gliomas through potentiating temozolomide, but not through radiosensitization: implications for the clinic, *J Neurooncol.* 2011 105 (2): 181-90.

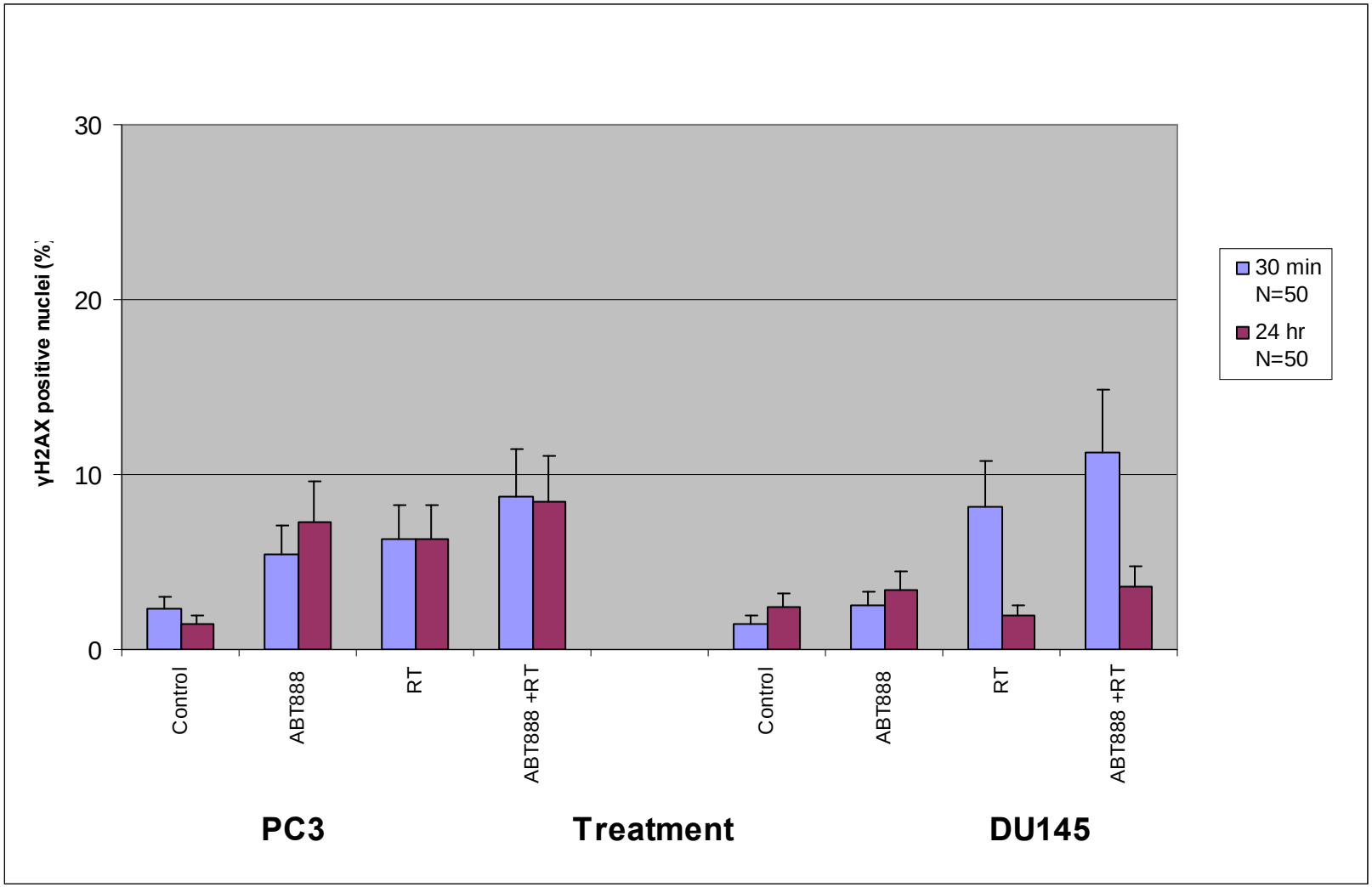
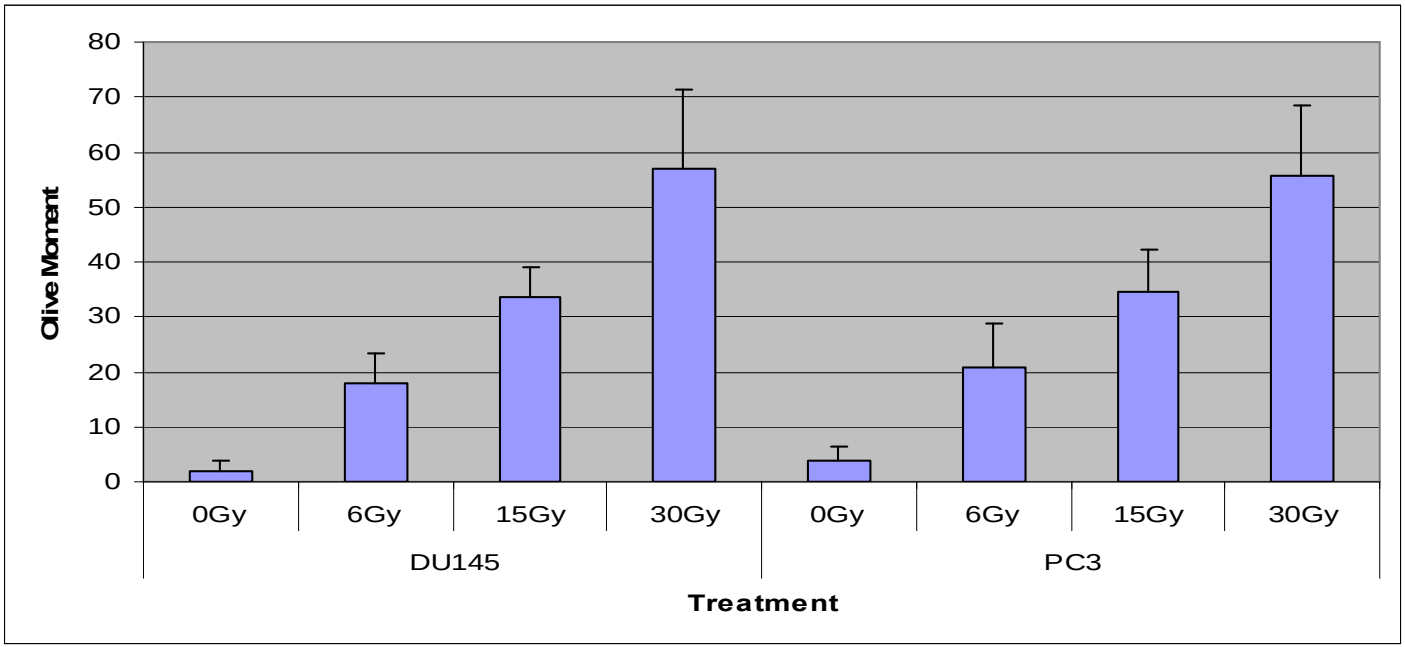


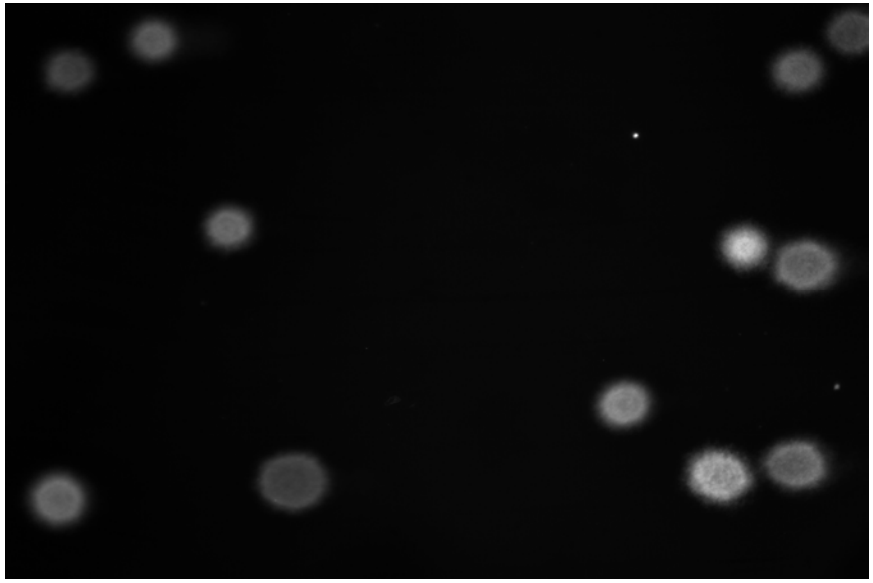
Figure 1. Effect of ABT888 (50 μM) and/or RT on induction of γH2AX foci in PC3 and DU145 cells. Cells containing nuclei with 3 or more γH2AX foci were classified as positive for DNA damage. 50 nuclei were counted for each treatment 30 min and 24 hr post RT (2 Gy). The mean +/- SEM from at least three independent experiments were obtained with three replicates per experiment.



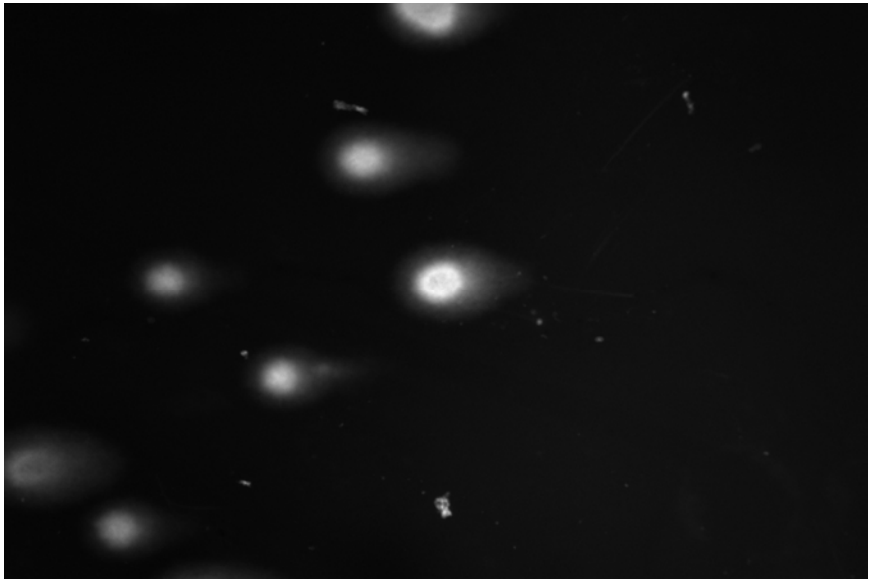
		N	Mean	Stdev
DU145	0Gy	48	2.01	1.85
	6Gy	42	17.82	5.45
	15Gy	54	33.61	5.49
	30Gy	40	56.91	14.35
PC3	0Gy	32	3.88	2.44
	6Gy	28	20.65	8.06
	15Gy	40	34.42	7.73
	30Gy	42	55.83	12.64

Figure 2. Comet Assay. Effect of radiation dose escalation on olive moment in DU145 and PC-3 cells. Cells were prepared for Comet assay according to instructions given in Trevigen kit.

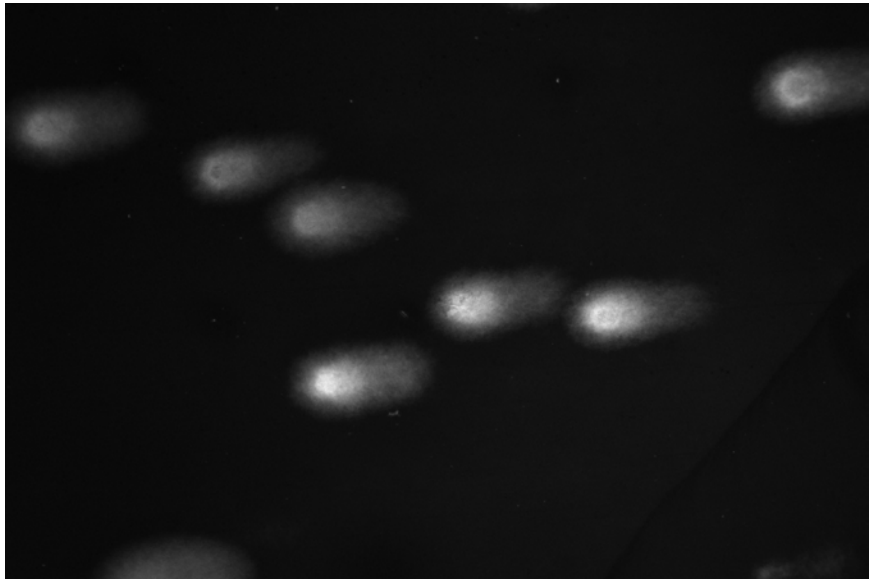
DU145, 0Gy



DU145, 6Gy



DU145, 15Gy



DU145, 30Gy

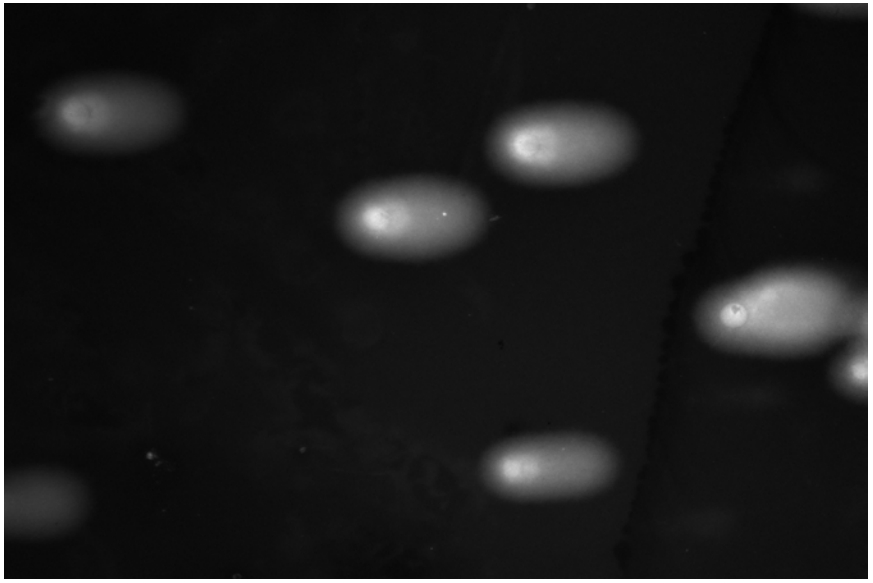


Figure 3. Comet images showing comet tails increasing with increasing dose of RT in DU145 cells.

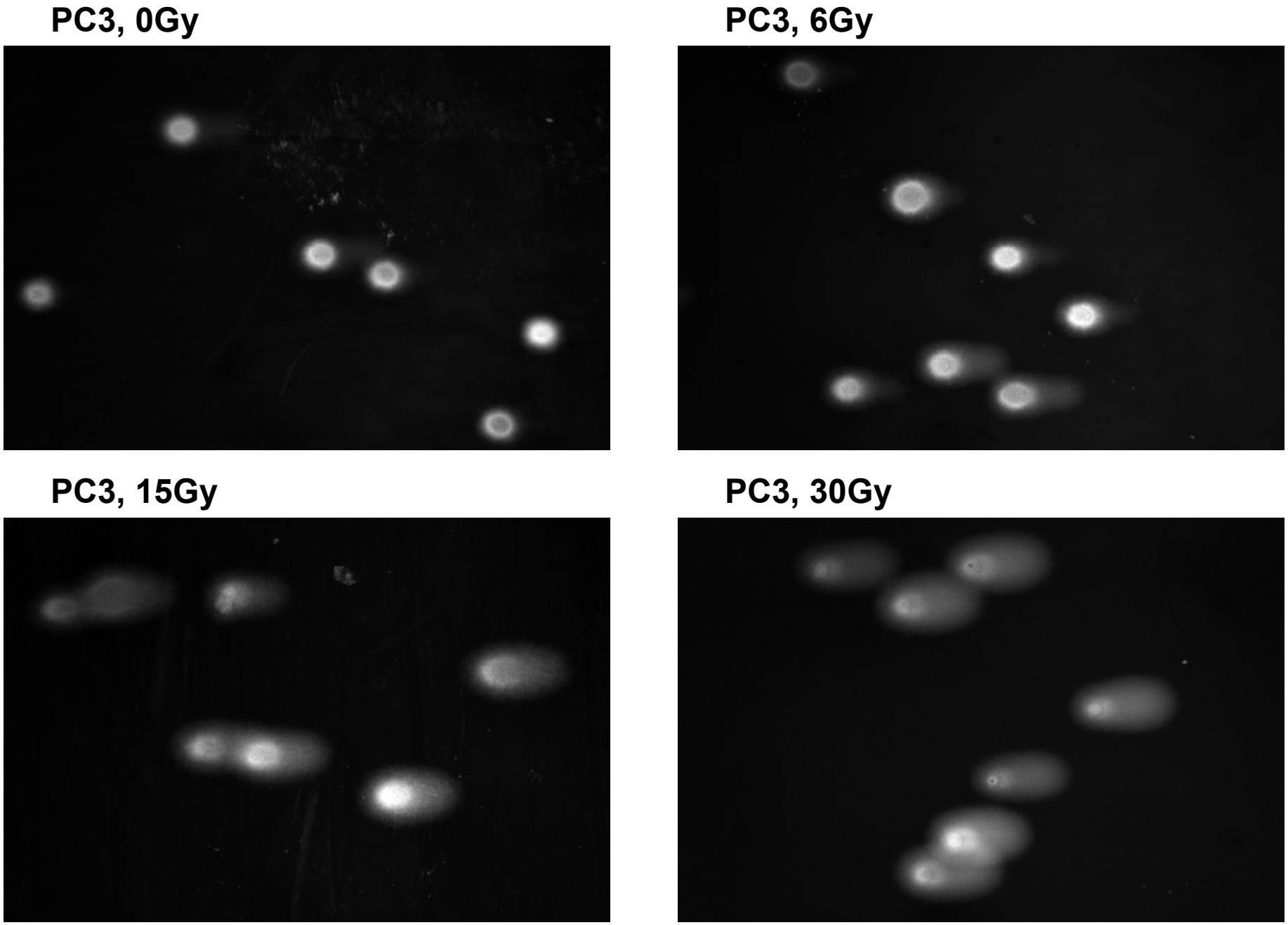
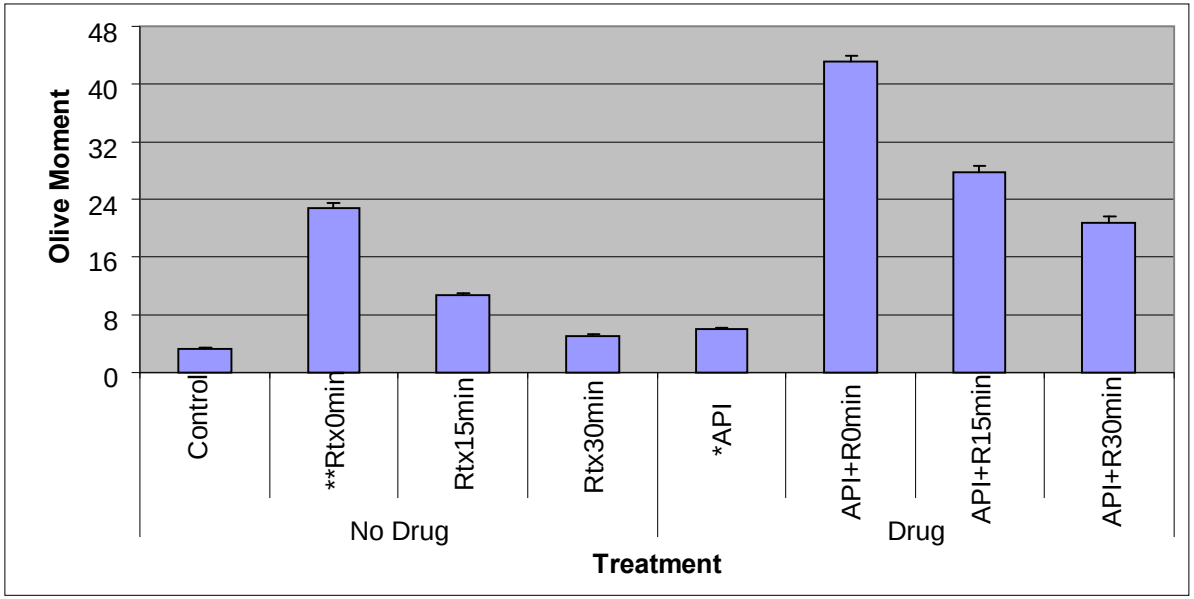


Figure 4. Comet images showing comet tails increasing with increasing dose of RT in PC-3 cells.

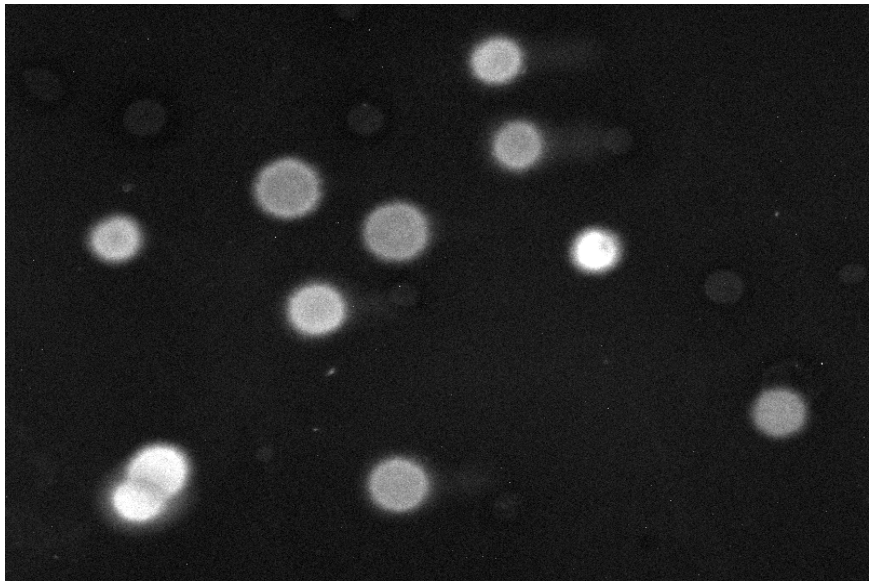


		N	Mean	Stdev	Stderr
No Drug	Control	56	3.27	1.43	0.191092
	Rtx0min	52	22.78	4.78	0.662867
	Rtx15min	59	10.73	2.29	0.298133
	Rtx30min	53	5.01	2.34	0.321424
Drug	API	65	6.04	1.66	0.205898
	API+R0min	36	43.11	4.69	0.781667
	API+R15min	42	27.84	5.42	0.836324
	API+R30min	48	20.80	4.99	0.720244

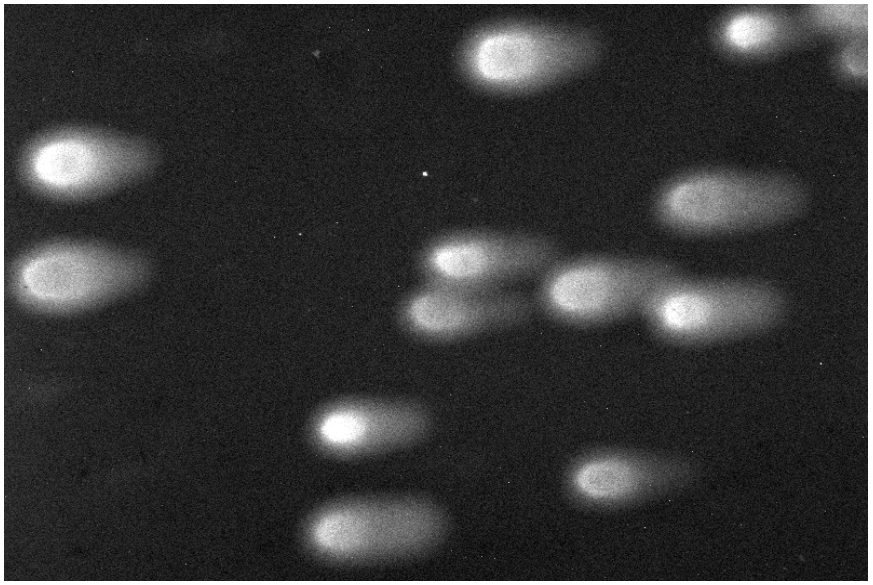
Figure 5. Comet Assay. Quantification of olive moment as a measure of kinetics of DNA repair in DU145 cells.

*API = Abbott PARP Inhibitor (ABT888) (50μM); **Rtx = radiation (15 Gy)

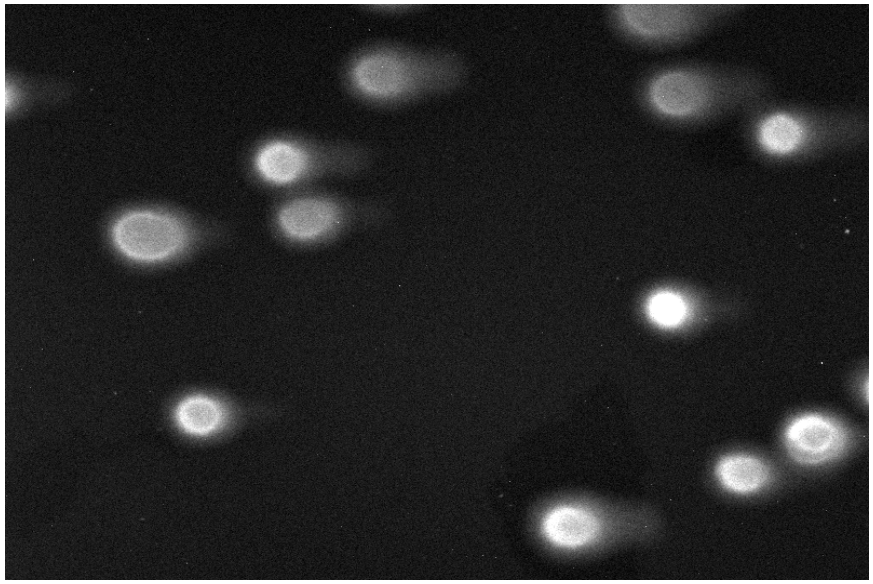
Control



Rtx, 0min



Rtx, 15min



Rtx, 30min

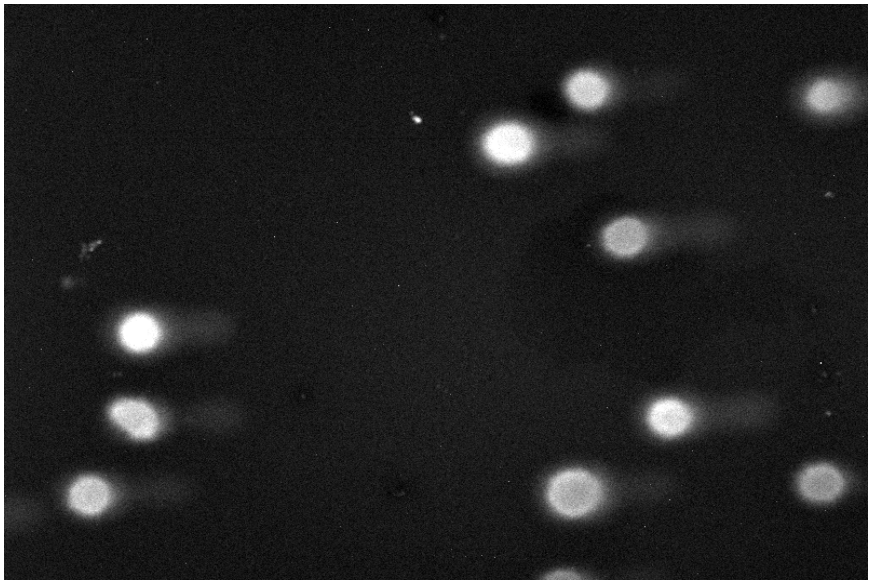
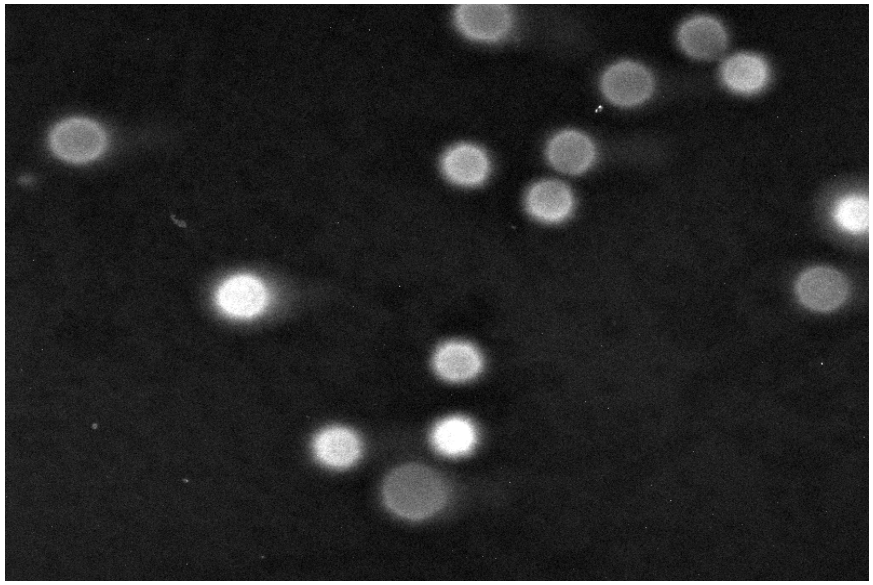
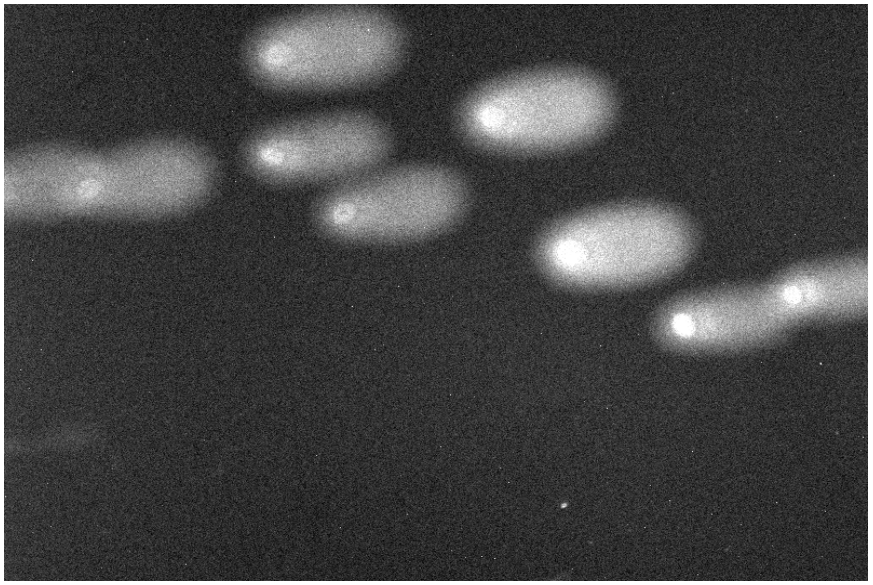


Figure 6. Comet tail images for DU145 cells

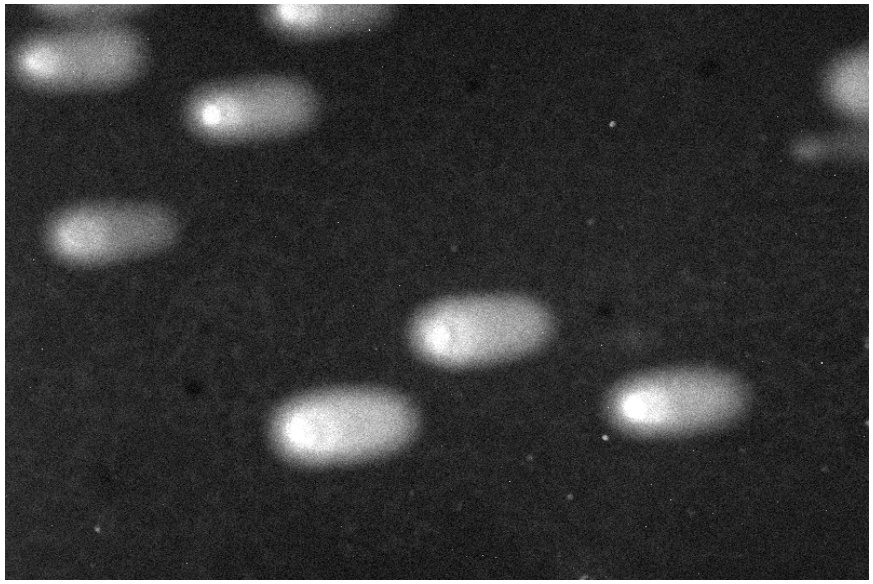
API (ABT888)



API (ABT888) +Rtx, 0min



API (ABT888) +Rtx, 15min



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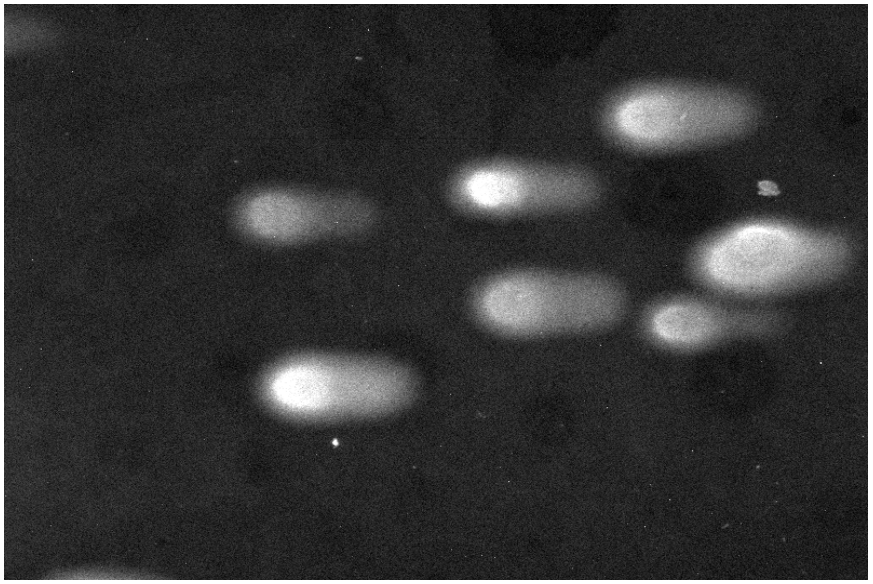
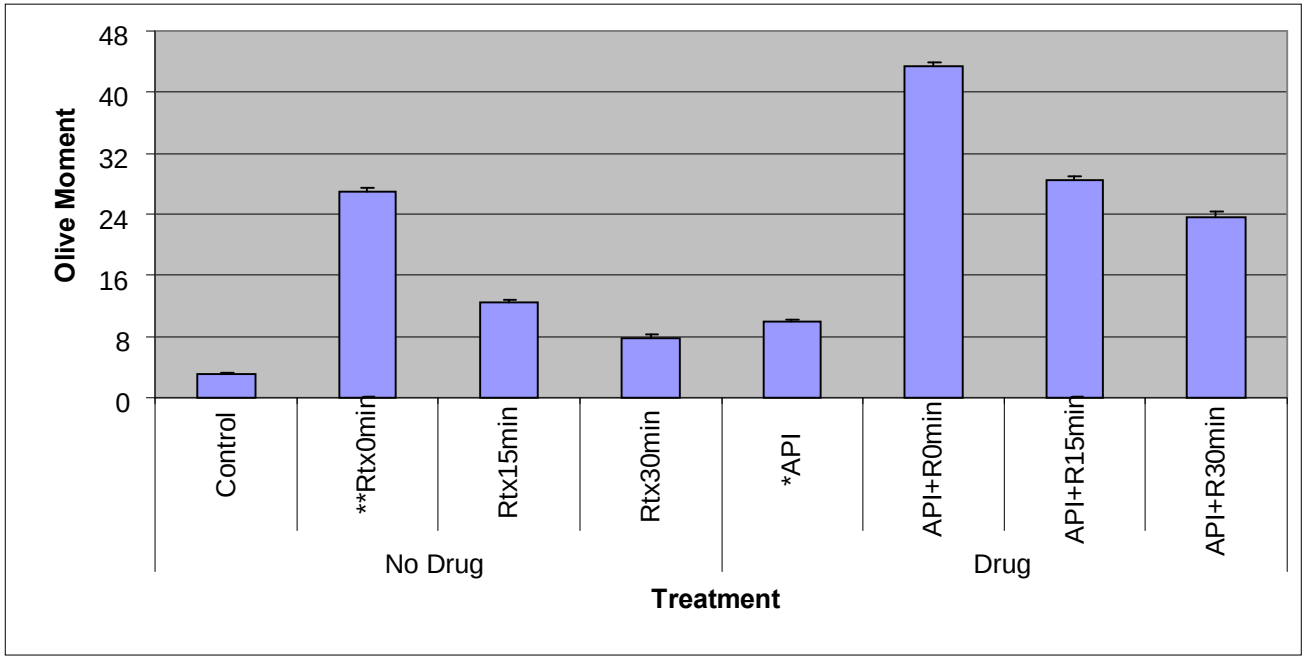


Figure 7. Comet tail images for DU145 cells.



		N	Mean	Stdev	Stderr
No Drug	Control	40	3.05	1.62	0.256144
	Rtx0min	40	26.90	3.70	0.585021
	Rtx15min	51	12.42	3.01	0.421484
	Rtx30min	30	7.82	2.86	0.522162
Drug	API	61	9.96	2.36	0.302167
	API+R0min	38	43.39	2.98	0.48342
	API+R15min	60	28.51	4.37	0.564165
	API+R30min	37	23.64	4.77	0.784183

Figure 8. Comet Assay. Quantification of olive moment as a measure of kinetics of DNA repair in PC-3 cells.

***API = Abbott PARP Inhibitor (ABT888 (50 µM); Rtx = **radiation (15 Gy)**

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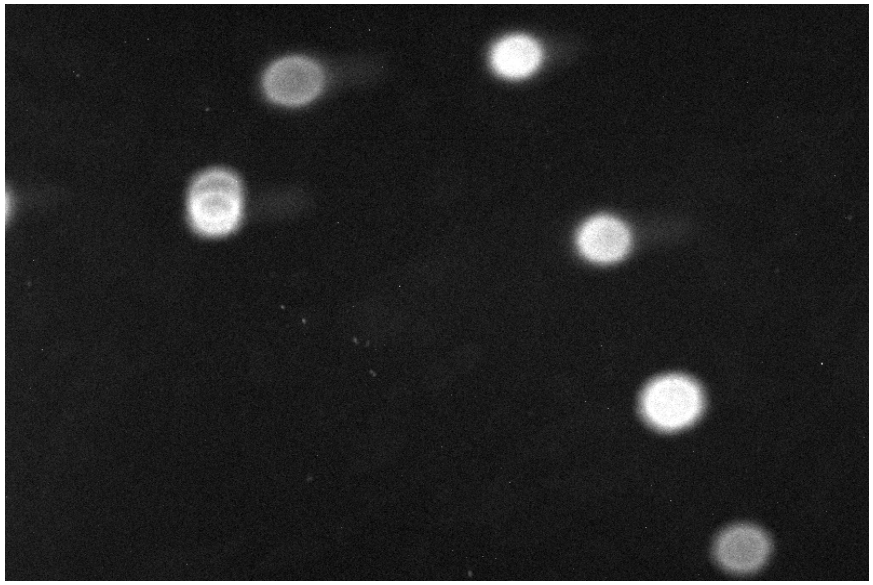
Control



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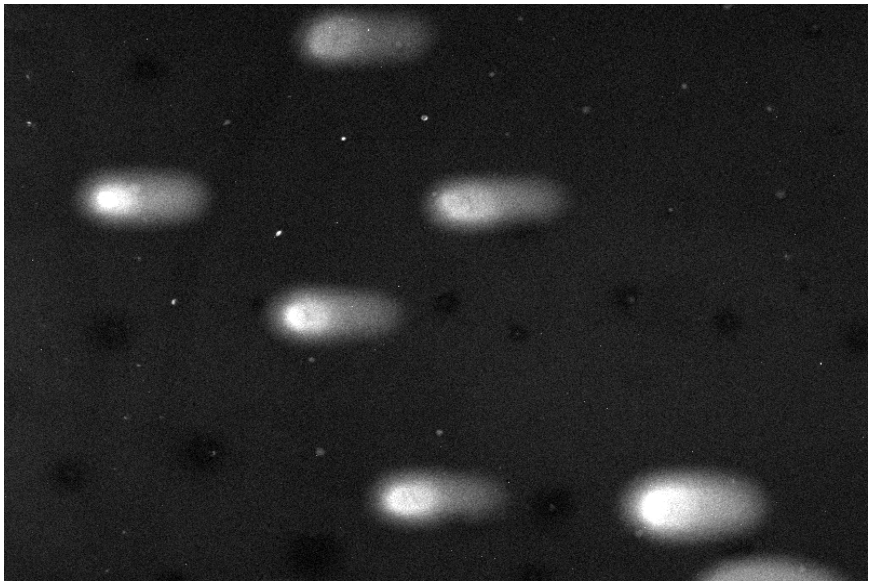

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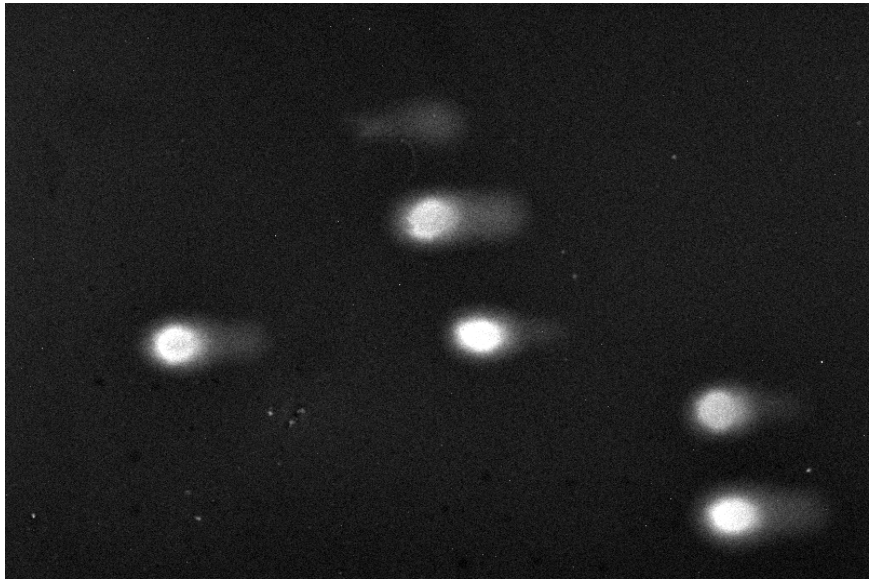

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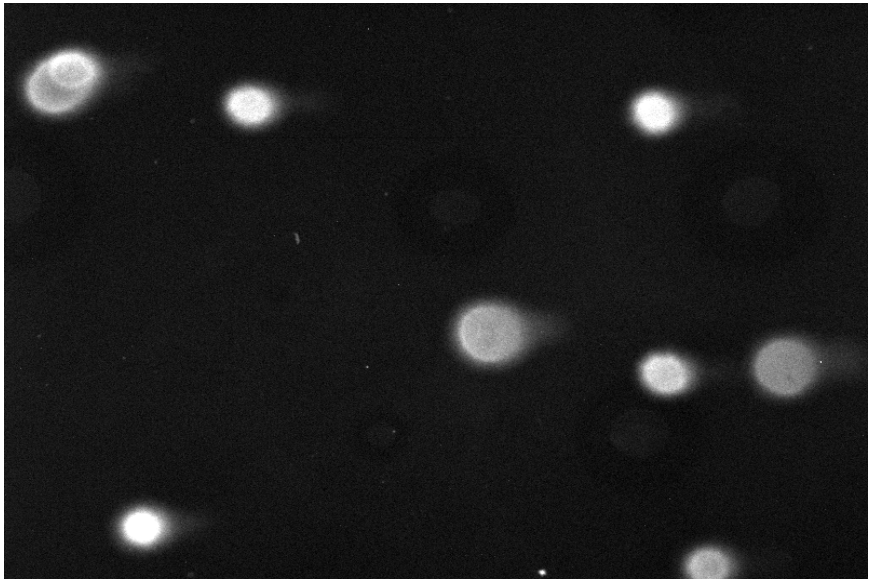
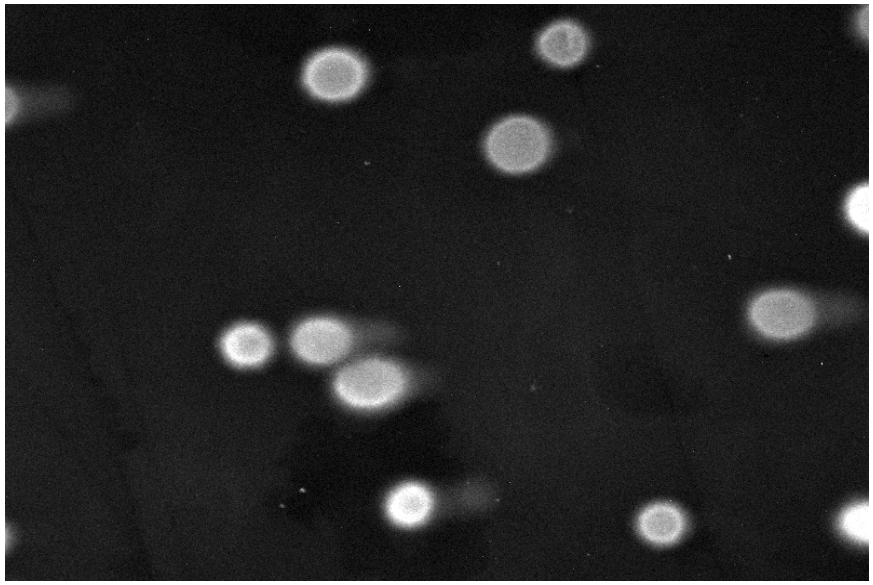
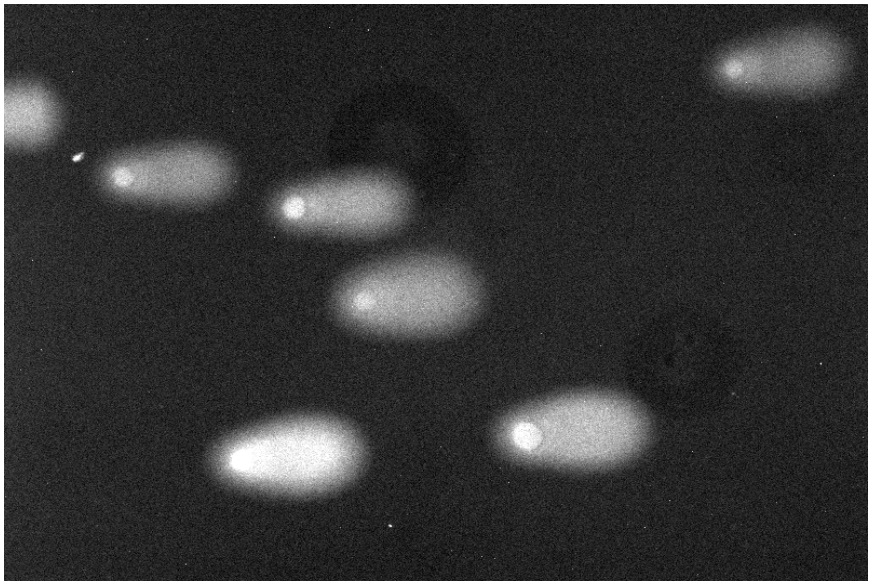


Figure 9. Comet tail images for PC-3 cells.

API (ABT888)



API (ABT888) +Rtx, 0min



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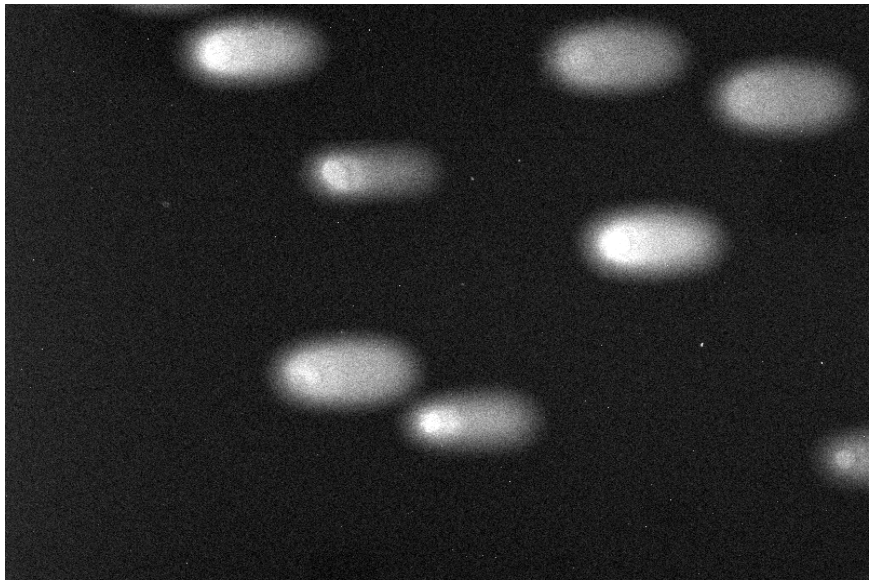

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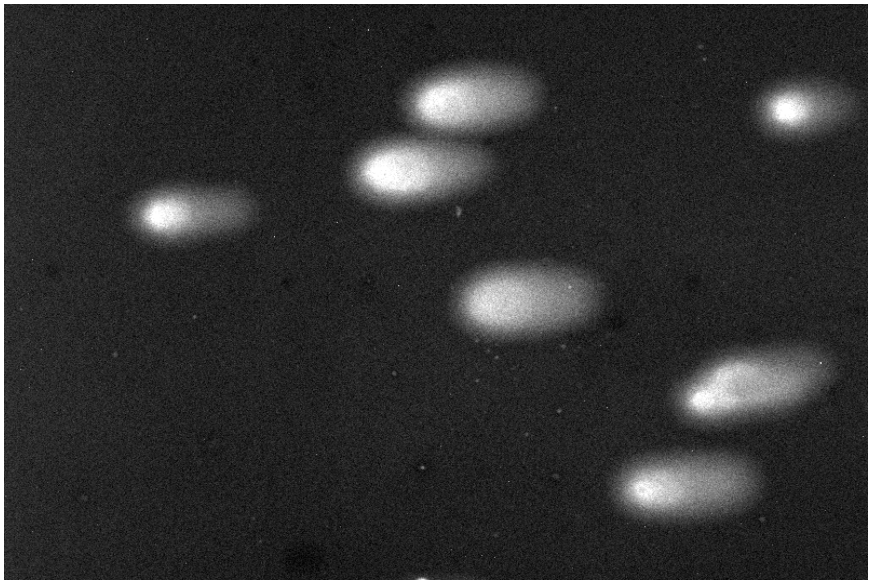
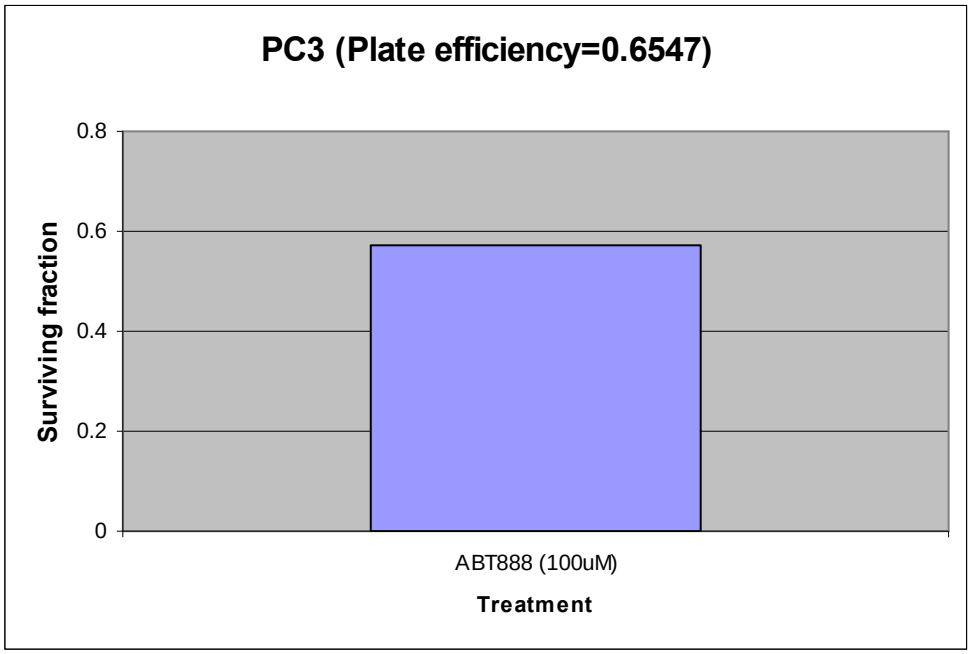


Figure 10. Comet tail images for PC-3 cells



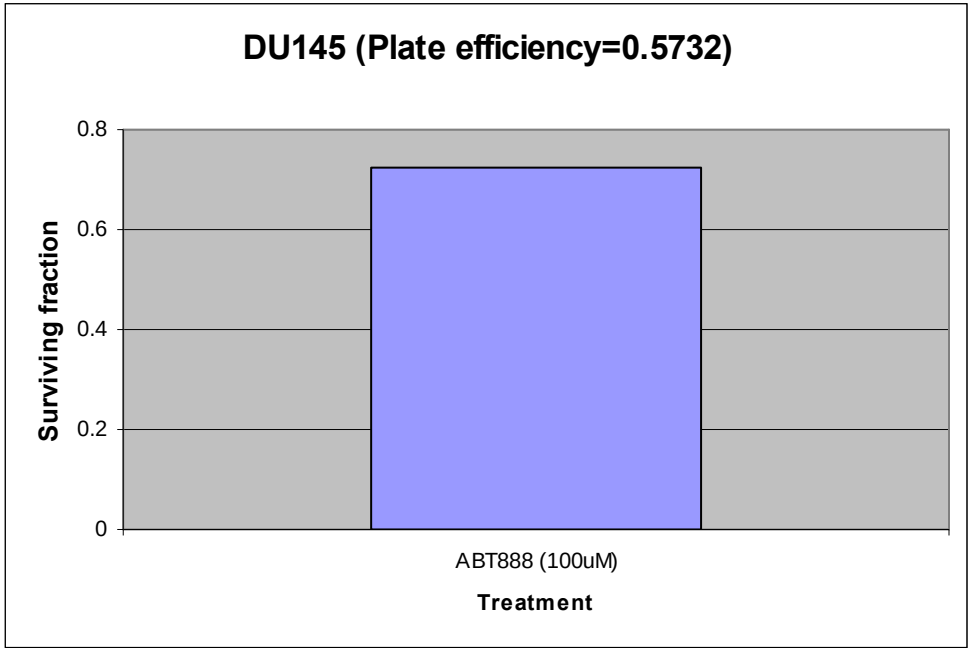


Figure 11. Clonogenic cell Survival after treatment with ABT888 for 24 hr in PC-3 And DU145 cells.

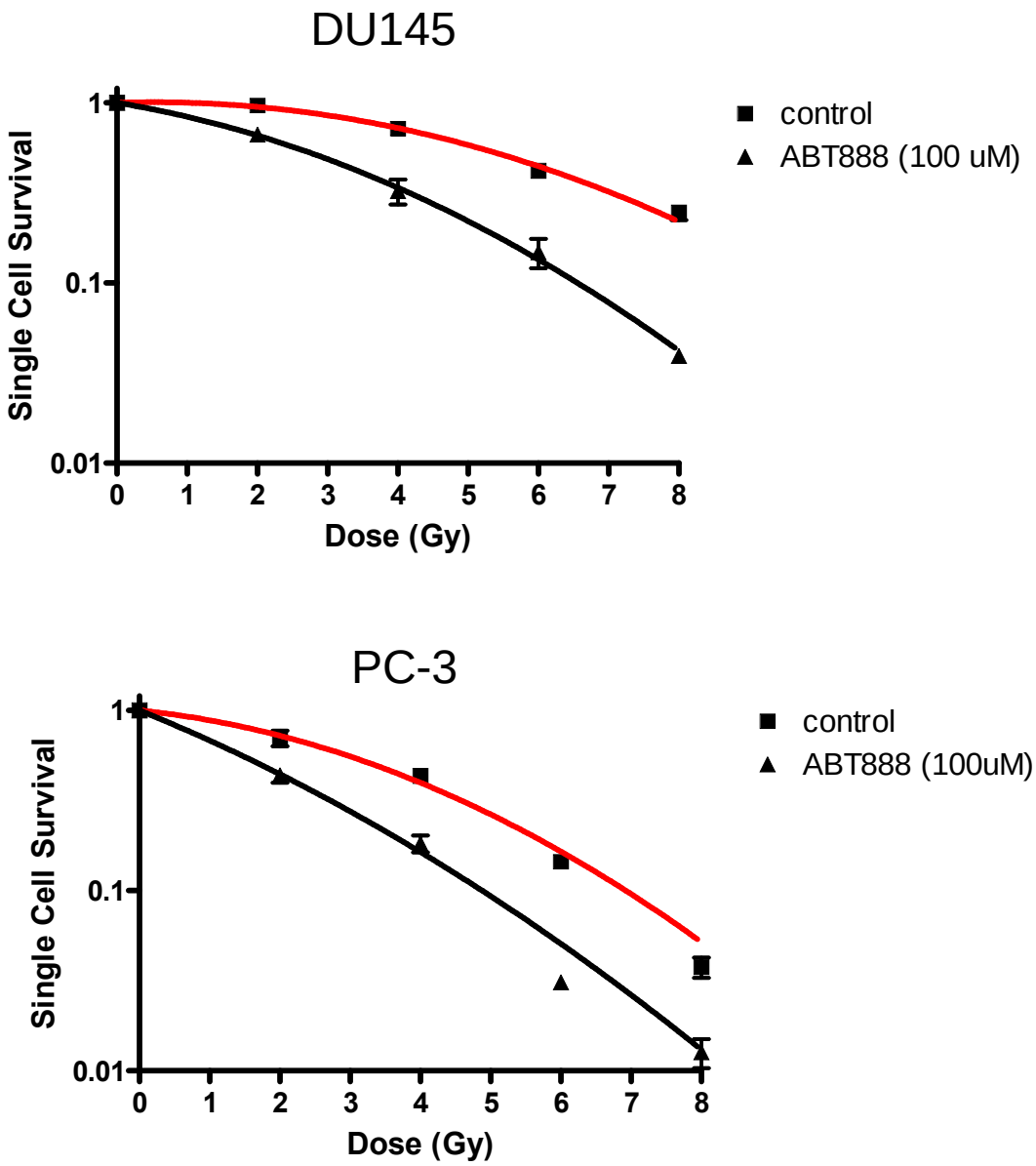


Figure 12. Clonogenic cell survival in DU145 and PC-3 cells after treatment with ABT888 for 24 hr.

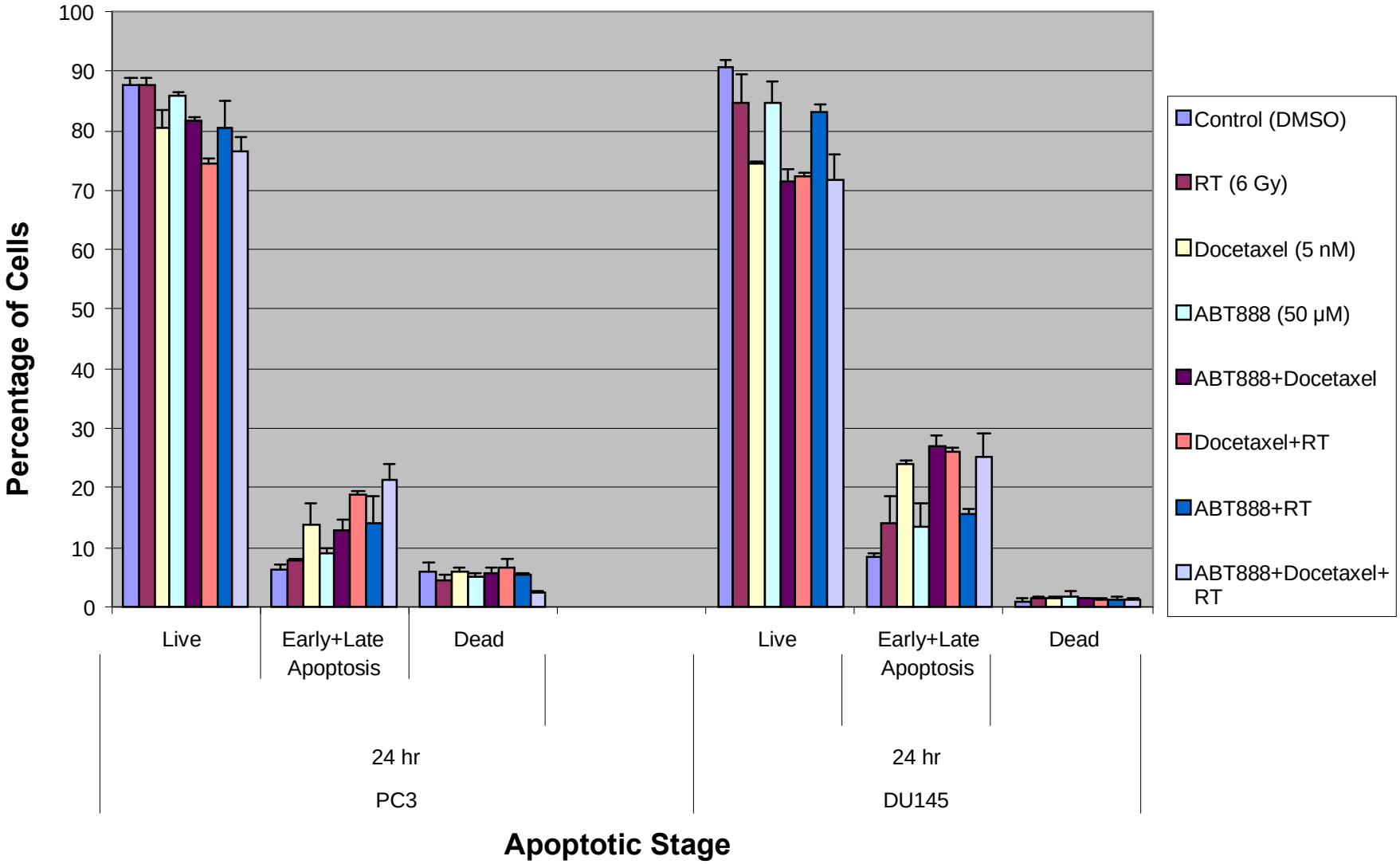
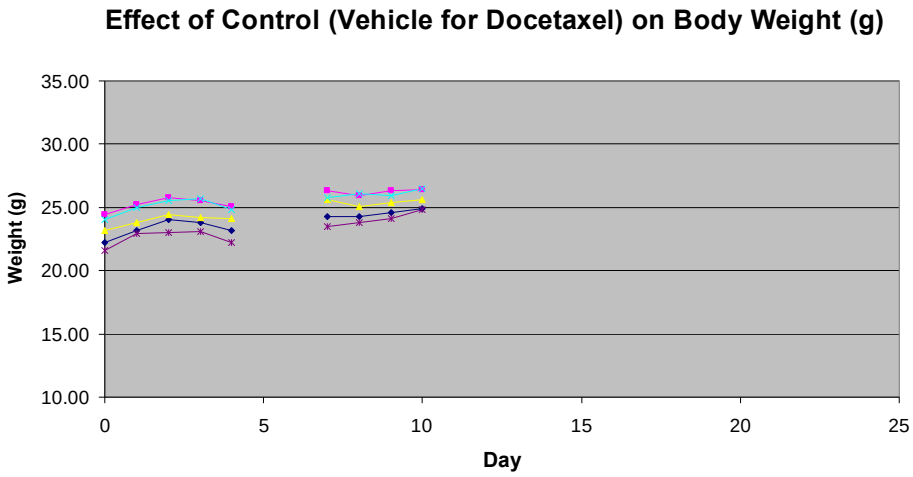


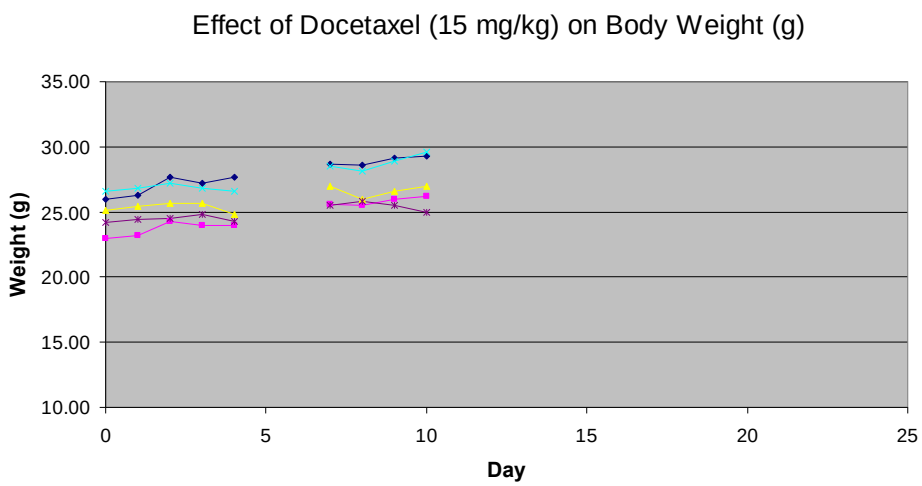
Figure 13. Effect of ABT888, Docetaxel and/or RT on induction of apoptosis in DU145 and PC-3 cells. The mean +/- SEM from at least two independent experiments were obtained with three replicates per experiment.

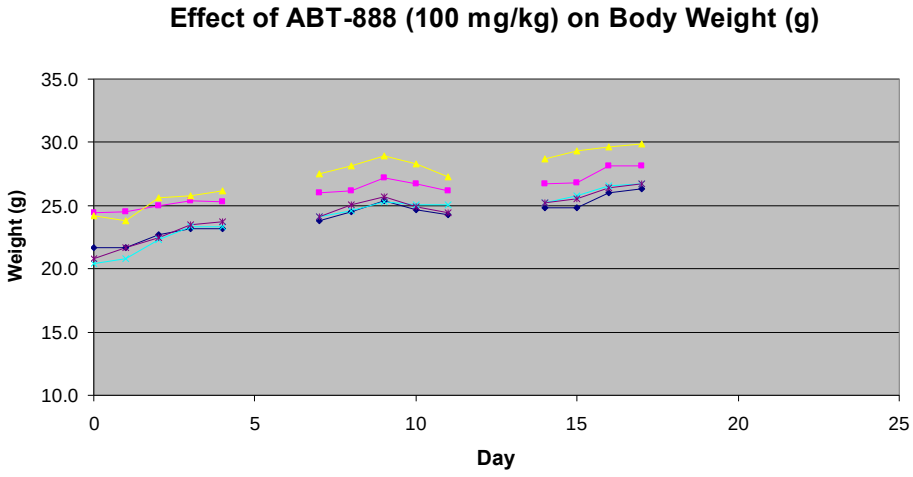
Treatment	*Apoptosis	
	DU145	**PC-3**
Control (DMSO vehicle)	8.3	6.4
RT	14.1	7.7
Docetaxel	24.1	13.7
ABT888	13.5	9.1
ABT888+Docetaxel	27.1	12.7
Docetaxel+RT	26.2	18.9
ABT888+RT	15.5	14.1
ABT888+Docetaxel+RT	25.1	21.2

*percentage of cell population undergoing early and late apoptosis

Figure 14. Tabulation of percentage of cells undergoing early and late apoptosis







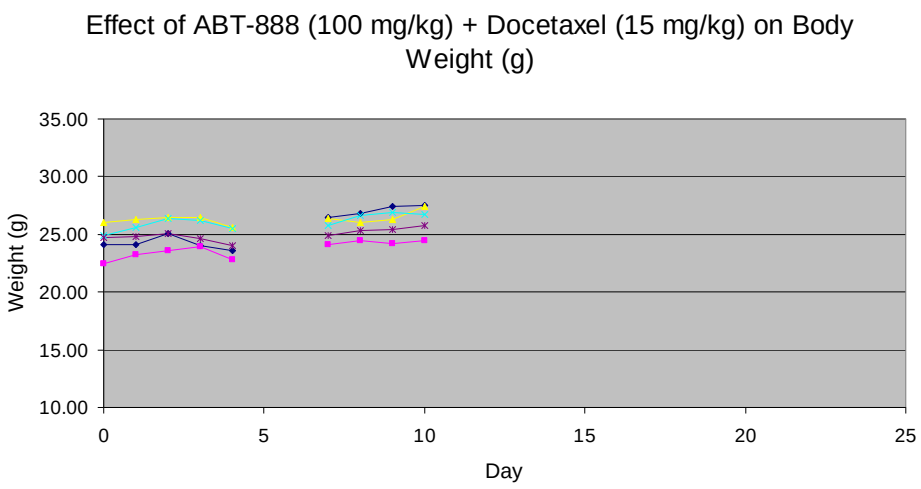


Figure 15. Effect of ABT888 and Docetaxel on non-tumored animals. (N=5 animals per treatment